Lipoprotein Lipase Immobilization onto Porous Polyvinyl Alcohol Beads

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SYNOPSIS

Water-insoluble proteases were prepared by immobilizing lipoprotein lipase (LPL) onto the surface of porous polyvinyl alcohol (PVA) beads by covalent fixation. The relative activity of the immobilized proteases was found to remain high toward small ester substrates, *p*-nitrophenyl laurate (pNPL). The relative activity of the immobilized LPL by cyanogen bromide (CNBr) method decreased gradually with the decreasing surface concentration of the immobilized LPL on the porous PVA beads. On the contrary, the immobilized LPL by hexamethylene diisocyanate (HMDI) method gave an almost constant activity for the substrate hydrolysis within the surface concentration region studied and gave higher relative activity (RA) than that by the CNBr method. The Michaelis constant, K_m, and the maximum reaction velocity, V_m, were estimated for the free and the immobilized LPL. The apparent K_m was larger for the immobilized LPL than for the free one, and V_m was smaller for the immobilized LPL. The pH, thermal, and storage stabilities of the immobilized LPL were higher than those of the free ones. The initial enzymic activity of the immobilized LPL maintained almost unchanged without any leakage and inactivation of LPL when the batch enzymic reaction was performed repeatedly, indicating excellent durability of the immobilized LPL. © 1993 John Wiley & Sons, Inc.

INTRODUCTION

Because the recovery yield and the reusability of free enzymes as industrial catalysts are quite limited, attention has been paid to enzyme immobilization[1] that offers advantages over free enzymes in choice of batch or continuous processes, rapid termination of reactions, controlled product formation, ease of enzyme removal from the reaction mixture, and adaptability to various engineering designs.[2-4] A concerted or sequential reaction of several enzymes is also obtainable by the use of mixed or stratified beds. Furthermore, the interest in the immobilized enzymes and their application to bioprocessing,[5,6] the analytical system,[7] and enzyme therapy[8] has steadily grown in the past decade. Thus, many approaches to the preparation of water insoluble enzymes have been explored in recent years[9-12] to study the enzyme reaction in biphasic systems similar to those existing *in vivo*.

However, effects of polymer supports on the activity of enzymes have not been studied in detail until now. In this study, lipoprotein lipase (LPL) is selected as a hydrolytic enzyme and the polymer support employed is porous polyvinyl alcohol (PVA)[13] beads that have very narrow pore size distribution. LPL catalyzes the hydrolysis of triacylglycerols in the form of chylomicrons to produce 2-monoacylglycerols and fatty acids[14] and also the hydrolysis of water-insoluble esters composed of fatty acids and alcohols with long chains.[15] LPL can be crystallized from *Pseudomonas fluorescens* and consists of two peptide chains linked by a disulfide bridge with a molecular weight of 33,000.[16,17] The effect of the method of covalent fixation of LPL onto the porous PVA beads on the hydrolytic activity of the immobilized LPL was studied. *p*-Nitrophenyl laurate (pNPL) was selected as a low molecular weight substrate for the enzyme reaction in this study. The stability and durabilities were also described for the immobilized LPL.

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EXPERIMENTAL

Materials

Porous PVA beads were prepared as follows.[13] Aqueous solutions were prepared from a commercial PVA powder with the degree of polymerization of 1,700 and the degree of saponification of 99 mol %. The PVA solutions having a polymer concentration mostly ranging from 10 to 20 wt %, were added dropwise to methylene chloride to make beads, and were frozen in a refrigerator kept at −20°C. Defreezing of the frozen PVA beads and the simultaneous crystallization of PVA phase were allowed to proceed slowly at 5°C. The resulting PVA beads were not soluble in water at room temperature but promptly dissolved in water when the temperature was raised to about 70°C. Figure 1 shows the scanning electron micrographs (SEM) of the porous PVA beads.

Crystallized LPL (EC 3.1.1.3) from *P. fluorescens* was kindly provided by Amano Pharmaceutical Co. (Nagoya, Japan). pNPL and other chemicals were purchased from Nakarai Tesque Co. (Kyoto, Japan).

Immobilization of LPL

Two covalent fixation methods were employed for immobilization of LPL onto the porous PVA beads, that is, hexamethylenediisocyanate (HMDI) and CNBr methods. Activation of PVA by HMDI was carried out according to the method described elsewhere[18] as follows. Porous PVA beads (500 mg) were placed in a three-neck round-bottom flask and purged with nitrogen gas. Distilled toluene solution (50 mL) containing 10 wt % of HMDI and 0.07 vol % of *di-n*-butyltin dilaurate was placed in the flask. The reaction mixture was gently stirred under nitrogen gas purging at 25°C. The activated beads were taken out of the reaction mixture at predetermined time intervals (generally 15 min of reaction time) and rinsed successively with distilled toluene and acetone. The beads activated by HMDI were immersed in LPL solutions (1.0 to 8.0 mg/mL in 0.1*M* PBS at pH 7.0). The immobilization reactions were allowed to proceed at 20°C for 5 h. The PVA–LPL conjugate beads were taken out of the solutions and washed successively with HCl aqueous solution of pH 3.0, 0.1% of aqueous sodium laurylsulfate (SDS) solution, and finally distilled water, each for 10 min.

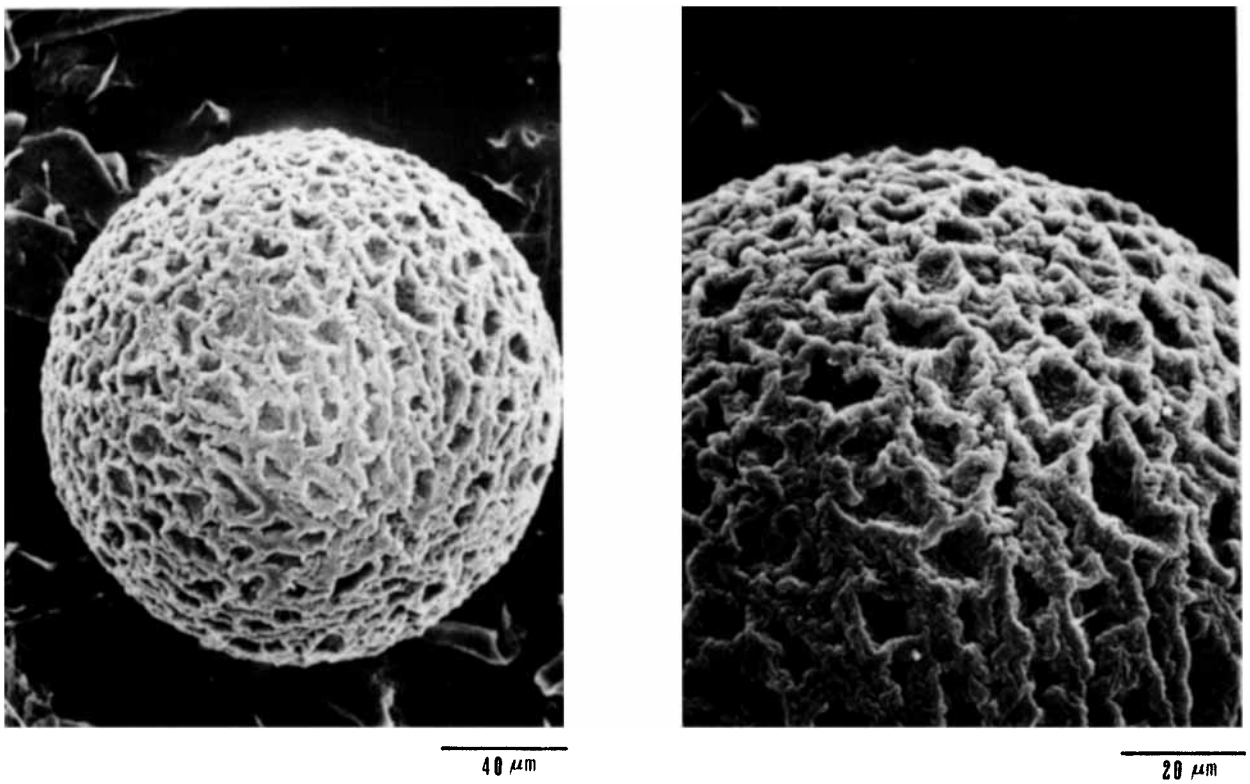

Figure 1 SEM of the surface of porous PVA beads.

Activation by CNBr was carried out referring to the procedure in the literature.[19] PVA beads (500 mg) were immersed in 100 mL 0.2*M* sodium carbonate at 4°C and gently stirred. To the mixture, 500 mg CNBr solved in 1.5 mL acetonitrile was added all at once. The reaction was allowed to proceed at 4°C for 1 h. After that, the activated PVA beads were taken out of the solution and washed successively with 0.1*M* sodium bicarbonate buffer, distilled water, and finally with 0.1*M* sodium bicarbonate buffer used for immobilization reaction. Immobilization of LPL onto the PVA beads activated by CNBr was carried out by immersing the activated PVA beads at 20°C in the LPL solutions at pH 9.0 (1.0 to 8.0 mg/mL) for 5 h. The conjugated beads were taken out and washed successively with 0.1*M* sodium acetate, 0.1% SDS, and distilled water, each for 10 min.

Amounts of LPL immobilized were determined by the ninhydrin method as follows. The LPL-immobilized PVA beads were hydrolyzed with 4 N HCl in an autoclave under 2.5 atmospheric pressure for 30 min. After that, the hydrolyzed solution was neutralized with 3.75 N NaOH and a ninhydrin solution was added. The absorbance at 570 nm was measured by a Hitachi model 200 spectrophotometer. In all the measurements, the PVA beads activated but not yet immobilized were used as blank to determine the amount of immobilized LPL.

Assay of LPL Activity

The hydrolytic activity of free and immobilized LPL was determined by using 0.01*M* pNPL in 0.05*M* PBS at pH 5.6. After incubating the reaction mixture under stirring for 20 min at 37.0°C, LPL was incubated by raising the temperature to 100°C for 5 min. The amount of *p*-nitrophenyl produced was determined by measuring the absorbance of the solution or the supernatant at 400 nm with the Hitachi 101 spectrophotometer. The absorbance was plotted against the LPL weight in the reaction mixture, and the initial slope of the curve was used to evaluate the activity.

The Michaelis constant, K_m, and maximum reaction velocity, V_m, were estimated by employing pNPL solution ranging from 1.0 to 10.0 m*M*.

Stability Measurements

The thermal stability of the immobilized LPL was evaluated by measuring the residual activity (ZA) of LPL exposed to various temperatures in 0.05*M* PBS, pH 7.4, for various periods of time. After heating the samples were quickly cooled and assayed for enzymic activity at 37.0°C immediately or after storage at 4°C. Storage before the assay (30 min to 48 h) did not alter the measured activities practically. The remaining activities were related to the original activities (assayed at 37.0°C without heating).

To determine the pH stability, the free and immobilized LPL was incubated in 0.05*M* PBS at 37.0°C including a definite amount of pNPL substrate and various pH regions for 20 min.

To evaluate durabilities of the immobilized LPL when repeatedly used, the dried immobilized LPL was washed in 0.05*M* PBS two times and then suspended again in a fresh reaction mixture to measure the enzymic activity. This cycle was repeated on the same sample. To check the possibility of any leakage of LPL molecules under washing, the amount of the immobilized LPL was determined after the last batch test. The storage stability of the free and immobilized LPL was evaluated by placing LPL in 0.05*M* PBS, pH 7.4, at 25°C for various periods of time and the activity was assayed using the above-mentioned techniques.

RESULTS AND DISCUSSION

Effect of Surface Concentration on Activity

The effect of the initial concentration of LPL on the saturated surface concentration of immobilized LPL was studied to make clear the most adequate initial concentration of LPL obtaining the highest surface concentration of the immobilized LPL on the porous PVA beads by CNBr method. As is seen in Figure 2, the amount of immobilized LPL is greatly influenced by the initial LPL concentration, at least in the low concentration level below about 4.0 mg/mL, but then seems to approach a constant value. Throughout the following experiments, the initial LPL concentration was kept to 5.0 mg/mL, unless otherwise mentioned.

Figure 3 illustrates the effect of the surface concentration of LPL immobilized onto PVA beads on the relative activity of pNPL hydrolysis. It is clearly seen that the relative activity (RA) of PVA-CNBr-LPL decreases gradually with the decreasing surface concentration of the immobilized LPL, especially at the surface concentration below 0.1 wt %, whereas PVA-HMDI-LPL gives an almost constant RA even at low surface concentrations, which is markedly higher than that of PVA-CNBr-LPL. This result may be explained in terms of structural deformation of the immobilized LPL molecules as illustrated in

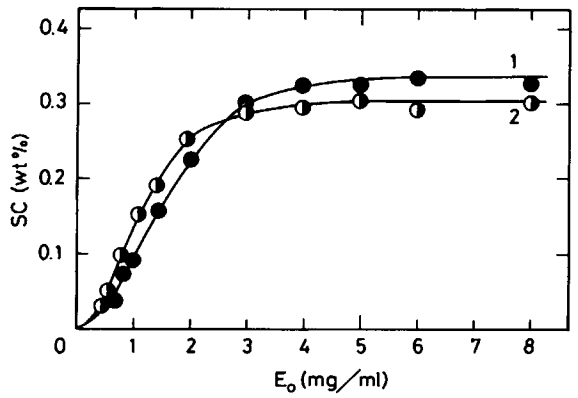

Figure 2 Effect of the initial concentration (E_o) of LPL on the surface concentration (SC) of LPL immobilized onto PVA beads (5 h): (1) PVA-CNBr-LPL and (2) PVA-HMDI-LPL.

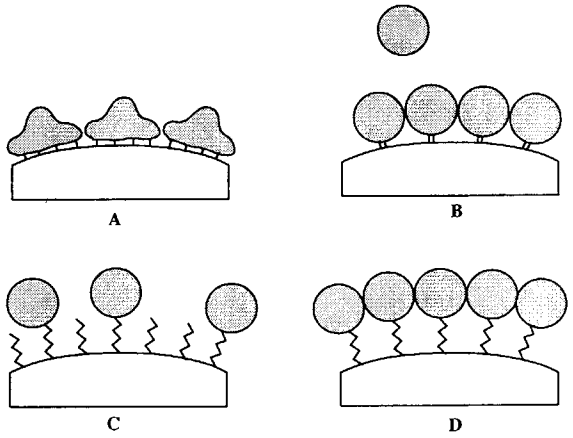

Figure 4 Schematic representation for the molecular state of LPL immobilized on the surface of PVA beads; (A) and (B) denote that of PVA-CNBr-LPL; (C) and (D) denote that of PVA-HMDI-LPL: (A) sparse immobilization and (B) dense immobilization of LPL molecules; (C) sparce immobilization and (D) dense immobilization of LPL molecules.

Figure 4. The covalently immobilized LPL by CNBr method may undergo strong deformation in the lower surface concentration region, whereas the immobilized LPL molecule by HMDI method must be protected from the heavy structural deformation even in the lower surface concentration region because of the spacer effect of HMDI. The low RA of LPL immobilized by CNBr method but having a high surface concentration may be ascribed to a reduced interaction with the substrate, as widely accepted.

Determination of Michaelis Constants

It seems interesting to analyze the enzymic hydrolysis with the immobilized LPL in the framework of the Michaelis–Menten mechanism, although the enzyme reaction takes place apparently in a heterogeneous state. In order to examine whether or not the rate of hydrolysis obeys the first-order kinetics with respect to enzyme concentration, a study was carried out by varying the enzyme concentration over a wide range. Figure 5 shows the observed results on pNPL hydrolysis by the free and the immobilized LPL. Clearly, the first-order behavior is observed with respect to the LPL concentration.

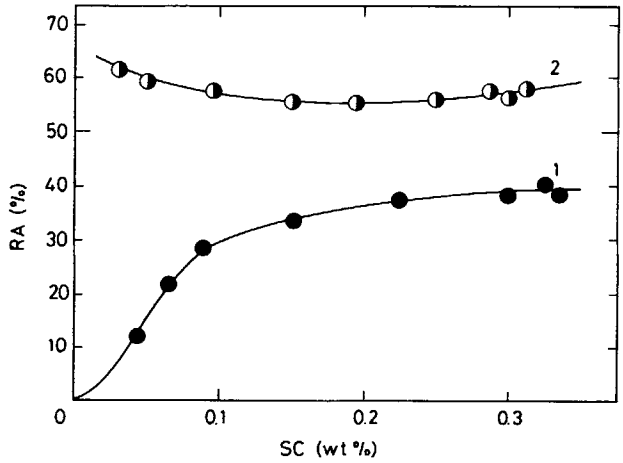

Figure 3 Effect of the surface concentration (SC) of LPL immobilized on the relative activities (RA) (hydrolysis; pNPL, pH 7.2, and 37.0°C). (1) PVA-CNBr-LPL and (2) PVA-HMDI-LPL.

Initial reaction rates were determined at different initial pNPL concentrations ranging from 1.00 to 10.0 m*M*. Figure 6 shows Lineweaver–Burk plots for the free and the immobilized LPL. The values of the K_m and the V_m for the free and the immobilized LPL on PVA beads are estimated from Figure 6 and tabulated in Table I. The apparent K_m values of the immobilized LPL were higher than that of the free one. It may be caused by the limitation of diffusion resistance. On the other hand, the V_m values of the immobilized LPL were lower than that of the free one, suggesting the RA of the immobilized LPL decreased in the course of the covalent fixation, especially with the case of the CNBr method.

Effect of pH on Activity

The pH effect on the RA of the immobilized and free LPL for pNPL hydrolysis was studied in 0.05 *M* PBS at 37°C in various pH regions. Figure 7 shows that the immobilized LPL has the same pH optimum

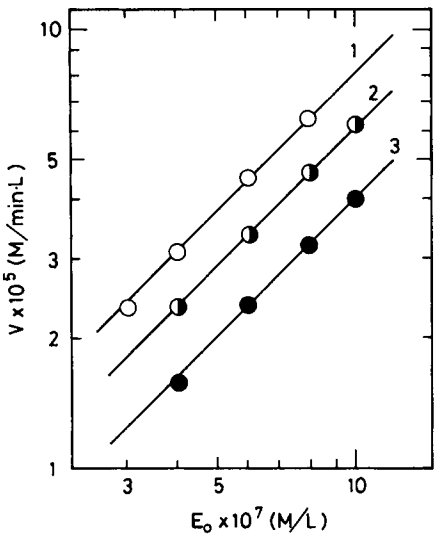

Figure 5 Effect of LPL concentration on the reaction velocity (hydrolysis; pNPL, 3.0×10^{-3} *M*, pH 7.2 and 37.0°C). (1) Free LPL; (2) PVA-HMDI-LPL; and (3) PVA-CNBr-LPL.

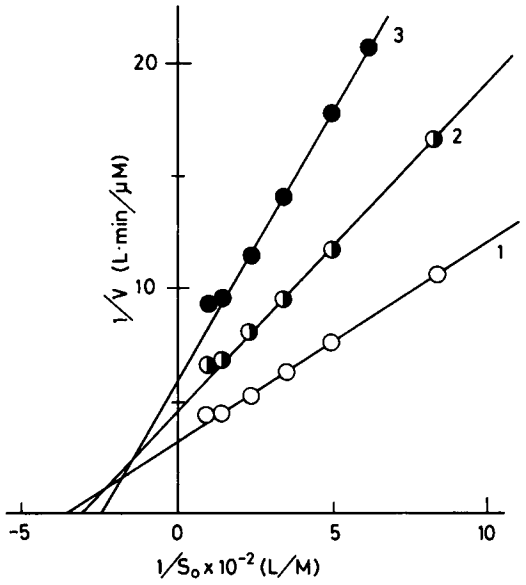

Figure 6 Lineweaver–Burk plots of 1/V vs. 1/S. (1) free LPL; (2) PVA-HMDI-LPL; and (3) PVA-CNBr-LPL.

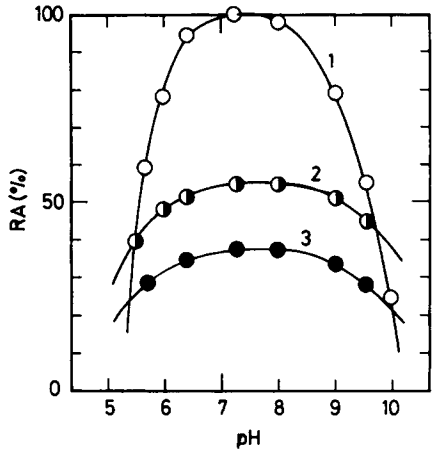

Figure 7 Effect of pH of the reaction medium on the relative activity (RA) of pNPL hydrolysis at 37°C: (1) free LPL; (2) PVA-HMDI-LPL; and (3) PVA-CNBr-LPL.

as the free one, but the pH profile is considerably widened because of diffusional limitations.[20]

Thermal Stability of Immobilized Enzymes

The thermal stability of immobilized enzymes is one of the most important criteria of their application. As is well known, the activity of the preparation of immobilized enzymes, especially in a covalently bound system, is more resistant against heat and denaturing agents than that of the soluble form.[21] The effect of temperature on the stability of the immobilized LPL in 0.05*M* PBS are shown in Table II. The immobilized LPL is more stable than the free one in the range of higher temperatures. For example, the residual activity (ZA) of the immobilized LPL by the CNBr method maintains almost 50% of the initial activity after treated at 60°C for 60 min, but that of the free LPL decreases to only 8% of the original value.

Storage Stability

Aqueous suspensions of the immobilized LPL could be stored at 4°C for 6 months without a significant

Table I Michaelis Parameters K_m and V_m

Sample Code	[*E*] (M/L)	K_m (M/L)	V_m (M/min · L)
Native LPL	4.0×10^{-7}	2.9×10^{-3}	3.3×10^{-5}
PVA-CNBr-LPL-1	4.0×10^{-7}	4.4×10^{-3}	1.6×10^{-5}
PVA-HMDI-LPL-1	4.0×10^{-7}	3.8×10^{-3}	2.5×10^{-5}

pNPL hydrolysis, pH 5.6 and 37.0°C.

Table II Effect of Heat Treatment on Residual Activity

Temperature[a] (°C)	Residual Activity (ZA) (%)		
	Free LPL	PVA-HMDI-LPL-1	PVA-CNBr-LPL-1
20.0	100	100	100
30.0	98	100	100
37.0	88	98	99
45.0	70	90	95
50.0	48	79	86
55.0	22	58	67
60.0	8	38	49

[a] Preheat temperature, 1 h at pH 5.6 in 0.1*M* PBS. ZA of pNPL hydrolysis at pH 5.6 and 37.0°C in 0.1*M* PBS.

loss of activity, whereas the corresponding free LPL lost more than 70% of their initial activity under the same condition. The higher stability of the immobilized LPL can be attributed to the prevention of autodigestion and thermal denaturation as a result of the fixation of LPL molecules on the surface of PVA beads. However, it is often pointed out that lyophilization of enzymes directly from the water suspensions is normally accompanied by loss of the enzymic activity. To ascertain this point for the immobilized LPLs, they were lyophilized with the conventional method from their aqueous suspensions. The results are given in Table III. Obviously, a very high ZA on pNPL hydrolysis is observed for the LPL immobilized by covalent fixation.

It is of interest to point out that there is a similarity between the thermal and storage stabilities to lyophilization. These findings can be accommodated in a general framework by considering the state of the covalent fixation between the carrier material and the enzyme molecules. It is reported that hydrophilic carriers such as Sephadex, Sepharose, and polyacrylamide yield enzyme derivatives of high lyophilization and thermal stabilities.[21-23] PVA beads belong to the hydrophilic carrier.

To examine the enzymic stability in the continuous reaction system under a rather drastic condition, effects of the storage in PBS, pH 7.4, at 37.0°C were studied for the immobilized LPL. The ZA at pNPL hydrolysis is given in Figure 8. It is apparent that the immobilized LPL by covalent fixation is much more stable than the free one. Again, the immobilized LPL by CNBr method shows more stable activity than that by the HMDI method that carries a certain spacer, despite the initial lower activity.

Table III Residual Activity (ZA) After Lyophilization

Sample Code	SC (wt %)	ZA (%) pNPL
Native LPL	—	80
PVA-HMDI-LPL-1	0.30	92
PVA-CNBr-LPL-1	0.32	96

Durability for Repeated Use

The durability of the immobilized LPL is also very important in applications because they are subjected to repeated hydrolysis reactions. Figure 9 illustrates the effect of repeated use on the ZA of pNPL hydrolysis by the immobilized LPL. The activity of the immobilized LPL by covalent fixation is seen to be retained without any definite loss, even if the batch reaction is repeated at least 10 times.

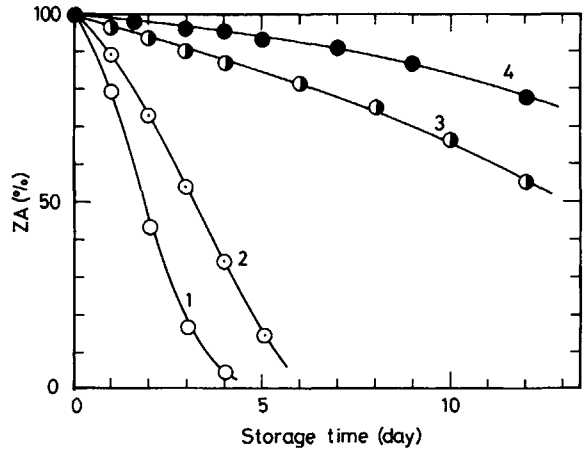

Figure 8 Effect of the storage time in PBS at pH 7.4 and 37°C on the residual activity (ZA) of pNPL hydrolysis at pH 7.2 and 37°C: (1) free LPL; (2) LPL adsorbed on PVA beads; (3) PVA-HMDI-LPL; and (4) PVA-CNBr-LPL.

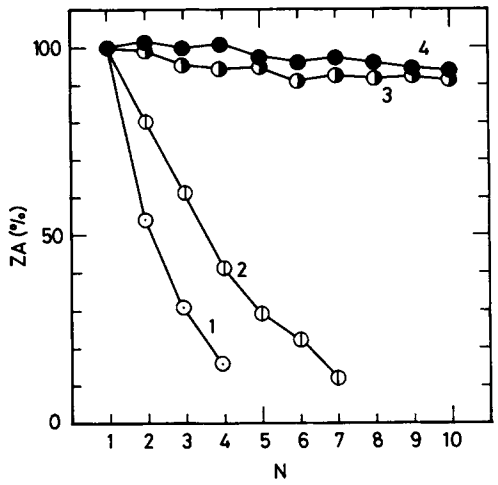

Figure 9 Effect of the repeated use on the residual activity (ZA) of LPL (hydrolysis: pNPL, pH 7.2, and 37.0°C). (1) LPL adsorbed on PVA beads; (2) LPL adsorbed on PVA beads and GA treatment; (3) PVA-HMDI-LPL; and (4) PVA-CNBr-LPL.

It was found that the amount of the immobilized LPL by covalent fixation after the last batch was equivalent to the original one within the experimental error in each cases, suggesting that no leakage of the immobilized LPL occurred under the repeated washing. This high stability is in marked contrast with the rather poor durability of the LPL that was immobilized by adsorbance on the porous PVA beads, even though glutaraldehyde treatment was performed.

CONCLUSION

The immobilized LPL onto the surface of porous PVA beads by covalent fixation gave rather high activity toward small ester substrates, pNPL. The RA of the immobilized LPL by CNBr method decreased gradually with the decreasing surface concentration of the immobilized LPL. On the other hand, the immobilized LPL by HMDI method gave an almost constant activity for pNPL hydrolysis with the surface concentration region studied. The apparent K_m values were larger for immobilized LPL than for the free one, and V_m values were smaller for the immobilized LPL.

The pH, thermal, and storage stability of the immobilized LPL were higher than those of the free one. The initial enzymic activity of the immobilized LPL by covalent fixation was maintained, almost unchanged, without any elimination and inactivation of LPL, indicating the excellent durability of the bound LPL molecules.

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